EFFECT OF SHORT-TERM ADMINISTRATION OF N-NITROSO COMPOUNDS ON LIVER HISTOLOGY AND ON PENTOBARBITAL-INDUCED SLEEPING TIME IN MICE

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ABSTRACT

All known carcinogenic nitrosamines tested with the exception of dipentylnitrosamine, increased pentobarbital sleeping time (PST) with 2 days of oral administration. Carcinogenic nitrosamines caused in addition, loss of glycogen, lipid accumulation, swelling of hepatocytes or hemorrhage and necrosis with lymphocytic infiltration in either centrolobular or periportal areas of the liver. Dipentylnitrosamine, which is a weak carcinogenic nitrosamine, resembled non-carcinogenic nitrosamines (dicyclohexylnitrosamine, N-ethyl-N-tert-butylnitrosamine, N-nitroso-prolineethylester) in causing significant reduction of PST. Dipentyl-nitrosamine increased the smooth endoplasmic reticulum of hepatocytes. Carcinogenic nitrosoureas did not produce visible effects in the liver histologically; 1-butyl-1-nitrosourea, and dimethylnitrosourea increased PST; 1-methyl-1-nitrosourea and 1-ethyl-1-nitrosourea shortened PST.

INTRODUCTION

The formation of nitrosamines and nitrosoureas in the stomach from the combination of nitrite with amines or certain drugs such as aminopyrine or pyribenzamine has recently been noted (Lijinsky and Greenblatt, 1972; Rao et al., 1973). Some of the N-nitroso compounds thus formed are known carcinogens, but others must be tested for carcinogenicity by using long and costly feeding experiments.

A procedure for indicating carcinogenicity of nitrosamines in periods as short as 4 days was previously reported by us (Nishie et al., 1972). Pretreatment of mice with known carcinogenic nitrosamines prolonged pentobarbital-induced sleeping time (PST); administration of non-carcinogenic nitrosamines shortened PST.

In the study reported here additional nitrosamines, as well as a few nitrosoureas, have been tested for their effect on PST and for ability to produce hepatic lesions as evidenced by histological examination.

MATERIALS AND METHODS

N-nitroso compounds were either synthesized by J. W. Pensabene or by Parish Chemical Co., Provo, Utah*, or were purchased commercially (Eastman Chemical Co. or Schuchardt Gmb. H. and Co.).

The compounds were dissolved in olive oil and administered per os (p.o.) to 18-21 g male Swiss Webster mice on 2 successive days. Control

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*Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

mice received only olive oil (5 ml/kg/day p.o.). Convulsant nitrosamines (N-nitrosopiperidine and N-nitrosohexamethyleneimine) were given in divided doses at 2 hr intervals to avoid convulsive death. On the third day duration of sleep time in response to intraperitoneal (i.p.) pentobarbital administration (80 mg/kg i.p.) was determined in control and treatment groups. Experiments were repeated with varying doses of N-nitroso compounds in order to determine the smallest dose required to produce a significant change in pentobarbital induced sleeping time (PST).

Histology

Liver tissues were removed and fixed in buffered 10% formalin, embedded in paraffin, sectioned and stained for glycogen by the McManus method. In some experiments, after fixation a portion of the tissue was treated with aqueous 1.0% osmium tetroxide and 2.5% potassium dichromate to stain lipids (Luna, 1968).

Electron Microscopy

For ultrastructural examination 1 mm^3 blocks of the left medial lobe of the liver were prepared as previously described (Nishie et al., 1972) and examined in an Hitachi HU-12 electron microscope.

RESULTS

Two day pretreatment with carcinogenic nitrosamines prolonged PST, and pretreatment with non-carcinogenic compounds shortened PST (Table 1). Dipentylnitrosamine (compound no. 14), which is a weak carcinogen (Druckrey et al., 1967) was an exception and shortened PST at all doses tested (300 to 3000 mg/kg/day). Carcinogenic nitrosoureas other than dimethylnitrosourea

(no. 7) either prolonged or shortened PST. Dimethylnitrosourea had a dual effect on PST, prolonging or shortening it depending on dose. Of the N-nitroso compounds which shortened PST, only nitrosoureas (nos. 7, 15, 16) caused loss of body weight.

Histological examination of livers from mice pretreated with carcinogenic nitrosamines revealed hepatocyte swelling accompanied by glycogen loss, accumulation of lipid, hemorrhage or necrosis with lymphocytic infiltration in either centrolobular or periportal and midzonal areas (Table 2, Fig. 1). Non-carcinogenic nitrosamines, carcinogenic nitrosoureas and dipentylnitrosamine did not produce visible changes in histologic appearance. The majority of the carcinogenic nitrosamines tested (13 out of 20) produced lesions in the centrolobular area, 3 compounds of 20 produced lesions only in periportal and midzonal areas. Dibutylnitrosamine and N-nitrosohexamethyleneimine produced controlobular lesions predominantly with occasional periportal damage. N-methyl-N-nitrosoaniline- and N-nitrosopiperidine- lesions were more frequent in periportal areas of the liver, but in certain animals, centrolobular lesions were produced.

Pretreatment with dipentylnitrosamine produced disruption of rough endoplasmic reticulum (RER) and proliferation of smooth endoplasmic reticulum (SER) (Fig. 2). Small, rounded clusters of both SER and RER were noted throughout the cell matrix, and the long, parallel vesicles of RER observed in control mice were absent.

Table 1. Effects of Two Oral Doses (2 Days) of Nitrosamines on Pentobarbital Sleeping Time (PST) and Body Weight in Mice.

No.	R1	R2 (R1, R2—N-N=O)	μ moles/kg/day (mg/kg/day)[a] 2 days	% Change Body Wt. 3 days	% Change PST over Control (p)[b]
		Compounds which prolonged PST			
1	methyl	ethanol[c]	192(20)	2.5	41(0.001)
2	methyl	pentyl	307(40)	-21	11(0.01)
3	N-nitrosomorpholine		430(50)	-14	61(0.02)
4	N-nitrosohexamethyleneimine		773(100)	-17	67(0.02)
5	ethyl	ethanol	1690(200)	0	59(0.01)
6	ethanol	ethanol	5900(800)	3.2	39(0.03)
7	methyl	$-CO-NH-CH_3$	2130(250)	-21	58(0.02)
8	butyl	$-CO-NH_2$	2750(400)	-21	35(0.05)
9	methyl	$-CH_2-COO-C_2H_5$	2730(400)	0	38(0.06)
		Compounds which shortened PST			
10	methyl	tert-butyl[c]	1508(175)	1	-39(0.05)
11	ethyl	tert-butyl[d]	1530(200)	0	-63(0.001)
12	cyclohexyl	cyclohexyl[d]	820(175)	4.6	-44(0.02)
13	N-nitrosoprolineethylester[d]		2900(500)	4	-47(0.02)
14	pentyl	pentyl	1610(300)	6	-55(0.001)
15	methyl	$-CO-NH_2$	480(50)	-12	-32(0.02)
16	ethyl	$-CO-NH_2$	640(75)	-7	-33(0.05)
7	methyl	$-CO-NH-CH_3$	1490(175)	-15	-32(0.05)
-	Phenobarbital sodium (reference)		20(5)	6.8	-37(0.05)

[a]Lowest doses of N-nitrosocompounds capable of producing significant change in PST.
[b]Probability level in Student t test.
[c]Nitrosamines for which carcinogenicity data is not available.
[d]Known non-carcinogens.

Figure 1.

1. 140 mg/kg/3 days p.o. dipropylnitrosamine caused swollen hepatocytes, loss of glycogen and lymphocytic infiltration in the centrolobular area c (160 X magnification, glycogen stain).

2. 140 mg/kg/3 days p.o. dipropylnitrosamine caused lipid accumulation in the centrolobular area c (160 X magnification, lipid stained in black).

3. 35 mg/kg/3 days p.o. N-methyl-N-nitroso-n-pentylamine caused glycogen loss in the periportal area p; centrolobular area c unaffected (160 X magnification, glycogen stain).

4. 35 mg/kg/3 days N-methyl-N-nitroso-n-pentylamine caused accumulation of lipid in the periportal area p (160 X magnification, lipid stained in black).

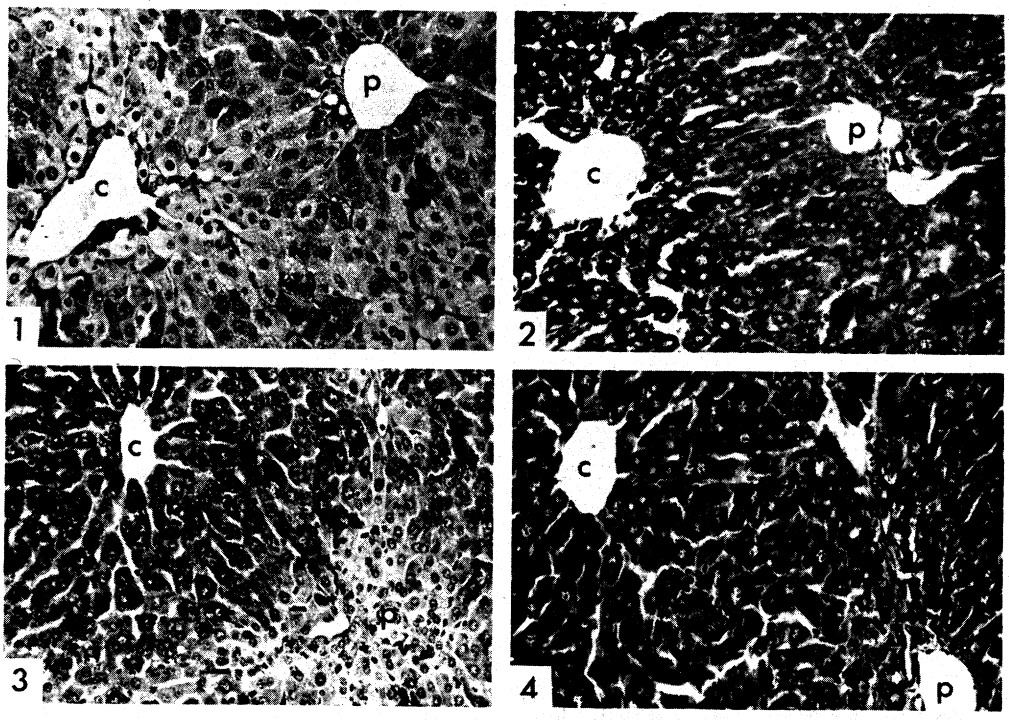
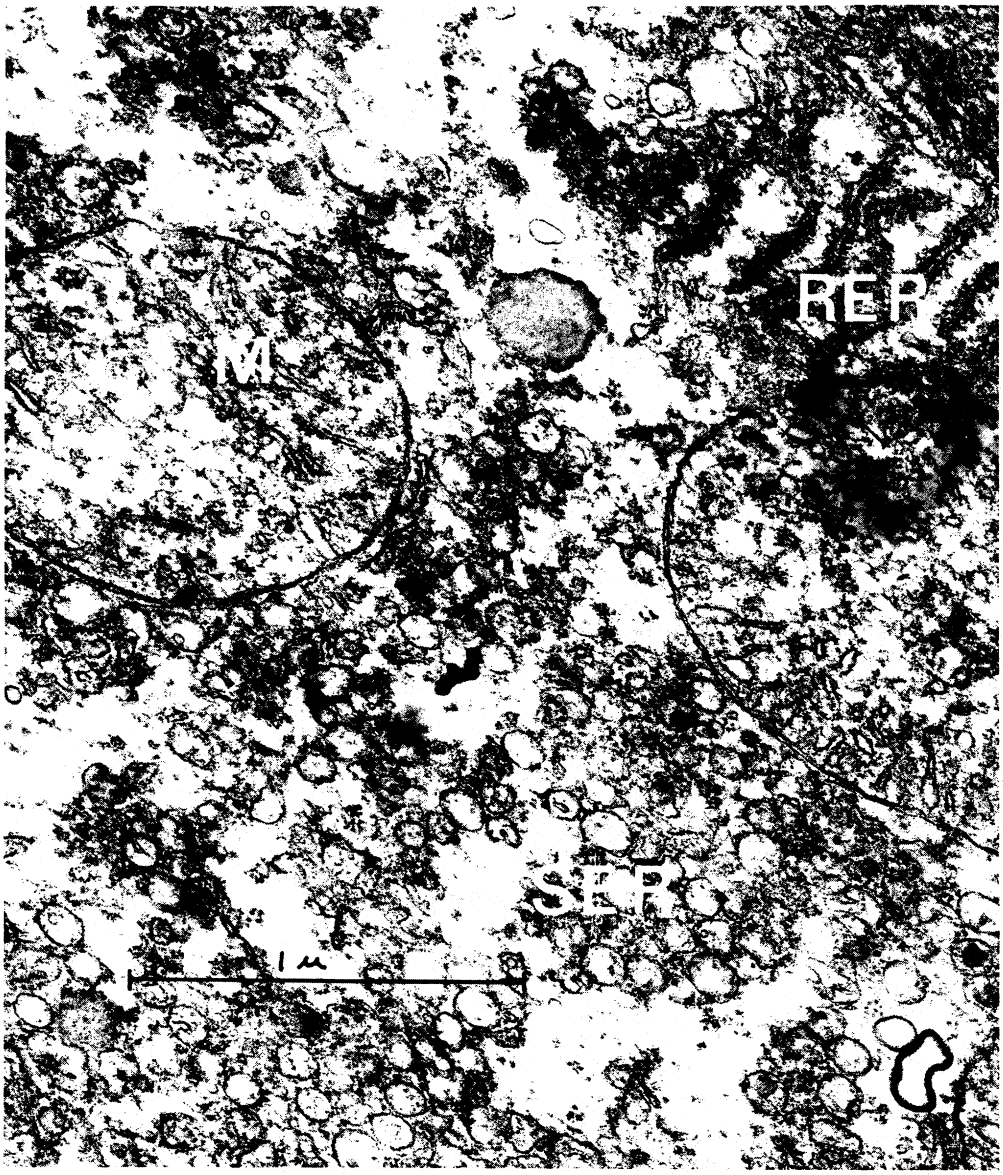


Table 2. Effect of Nitrosamines on Liver Histology

Nitrosamines	Dose (mg/kg x days p. o.)
Nitrosamines which produced lesions[a] in the centrolobular area.	
Dimethylnitrosamine	10 x 2
Diethylnitrosamine	20 x 3
Diethanolnitrosamine	800 x 2
Dipropylnitrosamine	100 x 2
N-methyl-N-nitrosoethylamine	25 x 4
N-methyl-N-nitrosoethanol	50 x 2
N-methyl-N-nitrosopropylamine	30 x 4
Ethylethanolnitrosamine	200 x 2
N-ethyl-N-nitrosopropylamine	30 x 4
N-ethyl-N-nitrosobutylamine	60 x 4
N-nitrosopyrrolidine	75 x 4
N,N'dinitrosopiperazine	100 x 4
N-nitrosomorpholine	50 x 2
Nitrosamines which produced lesions[a] in the periportal and midzonal areas.	
N-methyl-N-nitrosobutylamine	15 x 4, 20 x 2
N-methyl-N-nitrosopentylamine	15 x 4, 35 x 2
N-methyl-N-nitrosobenzylamine	30 x 2
Nitrosamines which produce lesions[a] in either centrolobular or periportal and midzonal areas of liver.	
Dibutylnitrosamine	(700-800) x 4
N-nitrosohexamethyleneimine	(80-125) x 2
N-nitrosopiperidine	(80-140) x 2
N-methyl-N-nitrosoaniline	(90-120) x 2

[a]Histological lesions in the liver: loss of glycogen, lipid accumulation, swelling of hepatocytes and/or hemorrhage and necrosis with cellular infiltration.

Fig. 2 - Electron micrograph of hepatocyte from dipentylnitrosamine treated (2000 mg/kg/day for 2 days) mouse. M, mitochondrion; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.



DISCUSSION

On the basis of the present report and the findings of Nishie et al. (1972), alterations in pentobarbital-induced sleeping time produced by prior administration of N-nitroso compounds appears to be a useful procedure for indicating carcinogenicity of nitrosamines, but not of nitrosoureas. Of 6 known non-carcinogenic nitrosamines studied to date, all shortened PST; of the 21 carcinogenic nitrosamines studied, only dipentylnitrosamine failed to increase PST. Carcinogenic nitrosoureas had varying effects on PST. Extrapolating the results of the study to 2 nitrosamines which have yet to be tested for carcinogenicity, methyl-ethanolnitrosamine would be expected to be a carcinogen and methyl-tert-butyl-nitrosamine a non-carcinogen.

The exception encountered in the failure of dipentylnitrosamine to increase PST may be due to the "weakly carcinogenic" nature of the compound as compared with other carcinogenic nitrosamines. Druckrey et al. (1967) found that a total dose of 48 g/kg of dipentylnitrosamine was required to produce tumors in rats. The periportal or centrolobular lesions typically produced by carcinogenic nitrosamines (Goodall et al., 1968, 1970; Kelly et al., 1966; Magee and Barnes, 1967) were not produced by dipentylnitrosamine, and the content of smooth endoplasmic reticulum was increased by dipentylnitrosamine, unlike the short-term effect of other carcinogenic nitrosamines (Nishie et al., 1972).

The predominantly hepatocarcinogenic nitrosamines produced acute lesions in the centrolobular areas of the mouse liver. On the other hand, the unsymmetrical nitrosamines reported to specifically produce

esophageal tumors (Druckrey et al., 1967; Druckrey, 1972) and tumors of the forestomach (Sander and Schweinsberg, 1972) produced acute lesions in the periportal and midzonal areas of mouse liver in the study reported here. The nitrosamines which produced either centrolobular or periportal lesions in mouse liver (Table 2), have been reported as carcinogenic to both esophagus and liver (Druckrey et al., 1967; Boyland et al., 1964; Garcia and Lijinsky, 1972; Goodall et al., 1968, 1970). Nitrosoureas exhibited no such correlation between organ specificity and site of hepatic lesions.

The study reported here suggests that differential hepatotoxicity of nitrosamines may be a useful indicator of the carcinogenic nature of the compounds. However, lack of correlation between hepatotoxicity and carcinogenicity of the closely related nitrosoureas indicates the impropiety of extending these observations to other classes of compounds. The mechanism of nitrosamine-induced alterations in PST and liver histology is yet to be determined, and precludes drawing any conclusions concerning relationship of acute toxicity of chemical compounds to ability to produce tumors.

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Physical Equilibria: Proteins

INTRODUCTION

Milk is a complex biological fluid, secreted by mammals explicitly for the nourishment of their young. Through the centuries, evolution has produced this stable, fluid, concentrated source of lipid, protein and carbohydrate. Because of its unusual stability (for a biological fluid) milk has become a valuable foodstuff, a commodity, yet many of the problems which arise in the processing of milk stem from the biochemical nature of its components. In dealing with skimmilk, the retention of the unique properties of the casein-protein complex during processing is of the utmost importance.

Chemically, the skimmilk system can be classified as a lyophilic colloid because the protein complexes of skimmilk, which constitute the dispersed phase, are in the correct size range, interact with and are stabilized by the solvent, and do not spontaneously coagulate. The milk protein complex is stable to the earth's gravitational field, yet can be separated from the liquid phase by centrifugation. Milk, then, can be considered a biocolloid; the properties of the dispersed phase (the casein-protein complex) and the dispersion medium (the milk serum) will be discussed.

THE CASEIN MICELLE

For better or for worse, the term "micelle" has been applied to the dispersed phase of milk, the casein-protein complex. The electron micrograph of Figure 9.1 shows a number of typical casein micelles. The nature of the casein micelle has been investigated in many laboratories, and with good reason, for this complex is the essence of a large number of problems encountered in dairy technology, whether it be the preservation of the stability of milk, the curd tension of cheese, or the production of a synthetic engineered food. Hence, understanding those forces

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*Revised from Chapter 9 in the 1st edition by T. L. McMeekin and M. L. Groves.

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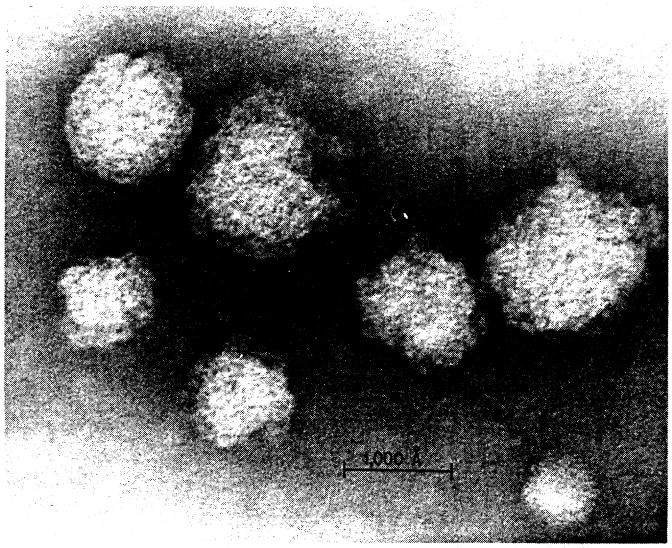


Courtesy of R. J. Carroll

FIG. 9.1. CASEIN MICELLES OF BOVINE SKIMMILK

Fixed in 1% glutaraldehyde and negatively stained with phosphotungstic acid.

which hold the casein micelle together, and *a posteriori* those forces which cause disruption of the casein protein complex, is of paramount importance.

Protein Components of the Casein Micelle

Until the 1930's the milk protein complex was considered to be composed of the rather "homogeneous protein," casein. Then, Linderstrøm-Lang[47] and Mellander[51] demonstrated the heterogeneity of bovine casein. The latter worker termed the electrophoretically distinct fractions α-, β- and γ- casein. From that time until the late 1950's, many methods of fractionation were developed and various casein fractions were isolated and characterized (see Chapter 3). The most significant fractionation was accomplished by Waugh and von Hippel;[102] when they discovered that the α-casein fraction is a mixture of α_{s_1}-casein and κ-casein. Indeed, a sample of casein from pooled milk, subjected to gel electrophoresis in urea and mercaptoethanol, yields up to 20 casein components. The demonstration of genetic polymorphism in the β-casein fraction by Aschaffenburg,[1] followed by the work of Thompson *et al.*,[91] on the genetics of the α_{s1}-fraction began to introduce a unifying concept to the field. The contention of Groves and Gordon[33] that the γ-, R-, S-, and TS-fractions are but degradation products of β-casein

leads one to speculate that the other minor casein fractions reported in the literature, such as m- and λ-caseins and the proteose peptone fraction,[93] are also degradation products of one or more of the major casein components. From all the work on the characterization of casein, three major components of the casein protein complex have been described, namely α_{s1}-, β- and κ-casein. Without a doubt the major protein of the casein complex is the α_{s1}-fraction. The exact margin by which this fraction exceeds β- and κ-casein seems to be open to debate depending in part on the method of quantitation. However, a good estimate obtained by several methods[70,71] would be α_{s1}, 50%, β, 33%, and κ, 15%.

The names of the various fractions used here are in accord with the A.D.S.A. committee, whose reports[72,87] have done much to order the field of milk protein nomenclature. Thompson[87] has recently reviewed the methods available for the detection of the various known genetic polymorphs of the milk proteins, and Farrell and Thompson[24] have reviewed their occurrence in various breeds and the possible biological significance of milk protein genetic polymorphism.

α_{s1}-Casein is the best characterized component of the casein system. It is a single-chain polypeptide, of known sequence, with 199 amino acid residues and a molecular weight of 23,600 daltons.[31,52,64] The molecule contains 8 phosphate residues, all of which exist as the phosphomonoesters of serine.[52,64] Seven of these phosphoserine residues are clustered in an acidic portion of the molecule bounded by residues 43 and 80 (the second fifth of the molecule from the N → C terminal end). This highly acidic segment contains 12 carboxylic acid residues as well as 7 of the phosphate residues; as postulated by Waugh,[104] it also contains the largest segment of the molecule's net negative charge. Theoretically, from a knowledge of the complete sequence, one can calculate the charge frequency, net charge, and hydrophobicity for various segments of the molecule. These data for α_{s1}-casein are presented in Table 9.1.

The hydrophobicity shown in Table 9.1 was calculated using the method of Bigelow,[6] and can be taken as a quantitative measure of the apolarity of a segment of a molecule or of the molecule itself. The data in Table 9.1 show noncoincidence of high charge frequency and apolarity in the segments shown; however, there exist local areas of charge surrounded by an apolar environment. The proline content of α_{s1}-casein is high;[31,52] these residues appear to be evenly distributed, and proline residues are known to disrupt helical and beta structures. Thus, the sequence data confirm the physical-chemical data, which indicated that the α_{s1}-molecule has little or no recognizable secondary structure such as α-helix or β-structure.[34] The high degree of hy-

Table 9.1

PROFILE OF THE α_{s1}-CASEIN MOLECULE DERIVED FROM ITS PRIMARY STRUCTURE[a]

Residues Considered	Net Charge[b]	Charge Frequency[b,c]	Average Hydrophobicity[c]
1 → 40	+3	0.25	1340
41 → 80	−22½	0.75	641
81 → 120	0	0.35	1310
121 → 160	−1	0.23	1264
161 → 199	−2½	0.14	1164

[a] Adapted from Grosclaude *et al.*,[31] Mercier *et al.*,[52] and Ribadeau-Dumas.[64]
[b] Some error as to assignment of these values may exist, since the exact placement of all amides is not known, serine phosphate = − 2, histidine = + ½.
[c] Calculated as described by Bigelow.[6]

drophobicity exhibited by the segment containing residues 100 → 199 is probably responsible, in part, for the pronounced self-association of the α_{s1}-casein monomer in aqueous solution.[73,75,82,104] This self-association approaches a limiting size under most conditions of ionic strength;[73,75,82,104] the highly charged phosphopeptide region can readily account for this phenomenon through charge repulsions.[104] It is noteworthy that while the self-association of α_{s1}-casein is mostly hydrophobic in nature, and hence temperature-dependent, some ionic bonding, as postulated by Schmidt,[73,75] must occur in the reaction in addition to hydrophobic interactions. At calcium-ion concentrations of 5 to 10mM,[90] α_{s1}-casein forms an insoluble precipitate. The solubility of α_{s1}-casein in aqueous Ca^{2+} solutions has been studied by Waugh *et al.*[103] and by Thompson *et al.*[90] With the exception of the rare genetic variant α_{s1}-A, the calcium solubility of α_{s1}-casein is temperature-independent.[90] Thus, the major protein component of milk is insoluble under normal conditions of pH, ionic strength, and temperature.

β-Casein is the second most abundant milk protein. The molecule is a single chain with 5 phosphoserine residues and a molecular weight of 24,500 daltons.[66,67] In aqueous solution, β-casein has been characterized as a random coil[56] with little or no secondary structure.[34] In aqueous solution, β-casein undergoes an endothermic self-association which reaches a maximum or limiting size depending upon the ionic strength.[58,79,104] An almost complete sequence is available for β-casein.[66,67] The proline content of β-casein is rather evenly distributed, which explains in part why the molecule lacks any secondary structure. The charge frequency, hydrophobicity and net charge for the various segments of β-casein are presented in Table 9.2. Analysis of these data indicates that β-casein is much more "soap-like" than α_{s1}-casein.

Table 9.2

PROFILE OF THE β-CASEIN MOLECULE DERIVED FROM ITS PRIMARY STRUCTURE[a]

Residues Considered	Net Charge[b]	Charge Frequency[b,c]	Average Hydrophobicity[c]
1 → 43	−16	0.65	783
44 → 92	−3½	0.13	1429
93 → 135	+2	0.23	1173
136 → 177	+3	0.07	1467
178 → 209	+2	0.06	1738

[a] Derived from the data of Ribadeau-Dumas *et al.*[64,67]
[b] Some error as to assignment of these values may exist, since the exact placement of all amides is not known serine phosphate = −2, histidine = +½.
[c] Calculated as described by Bigelow.[6]

The N-terminal portion of the β-casein molecule (residues 1 → 40) contains the phosphoserine residues and carries essentially all the protein's net charge, while the C-terminal half of the molecule (actually residues 136 → 209) contains many apolar residues (as demonstrated by its high hydrophobicity). The N-terminal concentration of charge and the highly hydrophobic C-terminal may account for the temperature-dependence of the self-association of β-casein[58] since hydrophobic interactions are temperature-sensitive.[43] Like α_{s1}-casein, β-casein is insoluble at room temperature in the presence of Ca^{2+} at concentrations below those encountered in milk. However, the precipitation from solution of β-casein is temperature-dependent, and the calcium-β-caseinate complex is soluble at 1°C up to 400 mM calcium-ion concentration.[90] Again, this temperature-dependence is probably due to the charge distribution of the β-casein monomer.

κ-Casein, the third major component of the milk protein complex, differs from α_{s1}- and β-casein in that it is soluble over a very broad range of calcium-ion concentrations.[102] It is this calcium solubility which led Waugh, upon discovering the κ-fraction, to assign to it the role of casein micelle stabilization.[102] It is also the κ-casein fraction that is most readily cleaved by rennin;[37,40,42] the resulting products are termed para-kappa and the macropeptide. It would appear that κ-casein is the key to micelle structure because it stabilizes the calcium-insoluble α_{s1} and β-caseins, and is the primary site of attack by the enzyme rennin. Ironically, κ-casein is at present the least well characterized fraction with regard to its primary structure and association properties. κ-Casein is unique because it is the only major component of the casein complex which contains cystine (or possibly cysteine). The occurrence of free sulfhydryl groups in the milk protein complex

has been reported[3] by some workers, but not by others.[41] Hence, the degree of disulfide bonding which occurs in κ-casein is uncertain, since no free −SH groups have been found in isolated κ-casein fractions.[41,42] Woychik *et al.*[107] reported the reduced molecular weight of κ-casein in 5*M* guanidine hydrochloride to be 17,000 to 19,000. Swaisgood and Brunner[81] reported a value of ≃19,000 for reduced κ-casein. The latter authors[80,81] reported a nonreduced molecular weight in the order of 60,000 for the lightest component, indicating at least a disulfide-linked trimer of the isolated κ-casein; but the weight average molecular weight of their preparation was ≃ 110,000. Cheeseman[17] studied the effect of the binding of the detergent sodium dodecyl sulfate on casein by gel filtration. He concluded that the majority of the κ-casein occurs as a disulfide-linked aggregate, which eluted at the void volume of Sephadex G-200, even in the presence of detergent.

κ-Casein is also the only major component of the casein complex that contains carbohydrate.[54] All the carbohydrate associated with κ-casein is bound to the macropeptide,[37,40,54] which is the highly soluble portion formed by rennin hydrolysis. In addition to being a glycoprotein, κ-casein contains one[37] or two[40] phosphate residues per reduced monomer and, as noted before, is soluble in Ca^{2+}, although it binds this ion.[21] Hill and Wake[37] postulated that κ-casein is an amphiphile or "soap-like" molecule on the basis of what is known of the primary structure of the macropeptide. This C-terminal one-fourth of the molecule, although it is quite hydrophilic and accounts for all the net charge of the κ-casein molecule, contains only a portion of the total number of charged residues. Jollès *et al.*[40] have isolated peptides from the supposedly hydrophobic para-kappa fraction, and have demonstrated that local areas of charge do exist in the para-kappa portion of the molecule. In addition, if κ-casein were a total amphiphile, as suggested, its physical associations should be highly temperature-sensitive. While its properties are temperature-dependent, it falls between β- and α_{s1}-casein in this respect.[7]

Forces Responsible for the Stability of the Casein Micelle

In 1929 Linderstrøm-Lang[47] postulated, as a result of his studies on casein, that the colloidal milk complex should be composed of a mixture of calcium-insoluble proteins stabilized by a calcium-soluble protein. The latter protein would be readily split by rennin, destabilizing the colloid and allowing coagulation to occur. As we have seen, such fractions do exist: α_{s1}- and β-caseins are indeed calcium-insoluble, while κ-casein is not only soluble in the presence of calcium ion, but is readily split by rennin. In addition, Waugh and co-workers[102,103]

have demonstrated that α_{s1}- and κ-casein complexes can be reformed from the isolated fractions as measured by sedimentation velocity experiments. Recently, Pepper[59] demonstrated this interaction of α_{s1}- and κ-casein by gel filtration, and studied the concentration-dependence of the interaction. The complexes formed by the interaction of the isolated α_{s1}- and κ-caseins aggregate to form simulated casein micelles upon addition of Ca^{2+} in 0.01*M* imidazole buffer, pH 6.7. As viewed by electron microscopy,[8] these synthetic micelles are virtually identical with fresh milk micelles except for their increased size. The precise mechanism of formation of the natural casein micelles is as yet uncertain, although several theories have been advanced based on the study of synthetic micelles; these theories will be reviewed later. In the course of the discussion of casein micelle structure and formation, a brief summary of the types of bonding forces responsible for the stabilization of protein structure will be given.

Hydrophobic Interactions.—One of the most significant contributions to our understanding of protein stability was made by Kauzman,[43] who elucidated the nature of hydrophobic interactions in proteins. These interactions come about because water exhibits a decreased entropy as a result of the occurrence of apolar amino-acid residues within the solvent. If these apolar residues are forced out of the water and into the interior of a protein molecule, where they can interact with other apolar groups, a small quantity of stabilization energy is gained per residue transferred from the solvent. Several model systems based on the energy of transfer of amino acids from water to ethanol have been studied and yield confirmatory results.[6,83]

These hydrophobic interactions are highly temperature-sensitive, being minimal below 5°C and maximal at higher temperatures. In a recent review article Klotz[44] pointed out that for proteins whose crystallographic structure is known, many apolar side-chains do exist fully or partially exposed to the solvent and therefore exhibit surface patches which are available for interactions with other protein molecules.

From the amino-acid analysis of the α_{s1}-, β- and κ-casein,[29,32,42] it is quite apparent that large numbers of apolar residues occur in these proteins. Furthermore, from the primary structures now available (Tables 9.1 and 9.2), it is clear that these hydrophobic residues are somewhat clustered for α_{s1}- and β-caseins, as well as for κ-casein.[37] According to the calculations of Hill and Wake,[37] the caseins rank among the most hydrophobic proteins of those tabulated by Bigelow.[6] It is not unexpected then, that the casein micelle should be stabilized by hydrophobic bonding. Several investigators[16,23,69,79] have noted that β- and κ-caseins, and α_{s1}-casein to some extent, diffuse out of the micelle

at low temperatures. As the temperature decreases, hydrophobic stabilization energy also decreases, and these molecules (β- and κ-casein) are able to diffuse out of the micelle. These observations are consistent with the known primary structure of β-casein (Table 9.2) and the postulated structure of κ-casein.[37] The interactions of β-casein are more temperature-dependent, which indicates that it is probably more "soap-like" than κ-casein. While all the authors cited above agree that β-, and to a lesser extent κ- and α_{s1}-caseins, can be removed from the casein micelle at 1°C, some question arises as to the exact amount released. Rose[69] reported high values for β-casein (up to 30%), while Downey and Murphy's values[23] (up to 15%) are lower. The latter workers, however, pointed out that the stage of lactation and health of the animal play a role in the percentage of cold soluble casein present. All those cited above concur that the α_{s1}-fraction does not diffuse from the micelle to as great an extent as the other two caseins.

The rare α_{s1}-A genetic variant, however, does exhibit highly temperature-dependent interactions. The α_{s1}-A gene is the result of the sequential deletion of up to 13 amino acid residues[29,52,89] bounded by residues 13 and 27, and the majority of these deleted amino acids are apolar.[89] The net result of this deletion is to bring the charged phosphorus-rich area closer to the N-terminal region, making this α_{s1}-genetic variant more like β-casein in its charge distribution, and the physical and solubility properties of α_{s1}-A mirror those of β-casein.[90] Thus, the stability of the casein micelle is due in part to hydrophobic interactions, although some ionic bonding must occur between the α_{s1}- and κ-caseins; the α_{s1}-A deletion probably does not permit the formation of the ionic bonds characteristic of α_{s1}-casein; as a result, micelles containing this protein are less stable to heat, cold, and processing conditions.

Dissociating agents, such as sodium dodecyl sulfate, guanidine-hydrochloride, and urea, all of which are thought to act primarily on hydrophobic interactions, tend to disrupt casein micelle structure in the same fashion, as evidenced by electron microscopy.[12] These solvents reduce the micelle to small subunits approaching 100 Å in diameter. The temperature-dependent properties of the hydrophobic interactions may also explain why milk can withstand moderate to high temperatures, but does not survive extremely low temperatures, such as freezing or ultrahigh temperatures.

Electrostatic Interactions.—It has been pointed out[9,44] that essentially all of the ionic side-chains in the proteins, whose crystallographic structure is known, are fully exposed to the solvent. Thus ionic bonding between negatively charged carboxylic acid residues and positively charged groups contributes little to the stability of a monomeric pro-

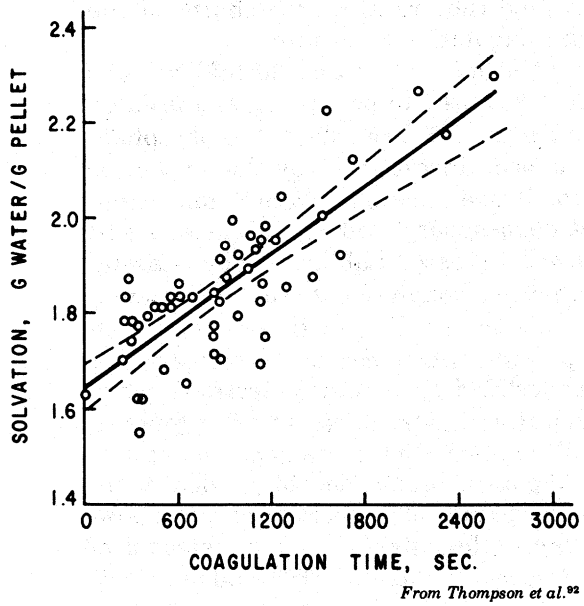
tein. Notable exceptions to this rule may occur when an ion pair can be formed within a hydrophobic environment;[83] the interactions of subunits of a protein may provide just such an environment. Physical-chemical evidence for the role of ionic bonding in subunit interactions is abundant, while crystallographic evidence is limited to hemoglobin,[26] although several subunit enzymes are currently under study.[35] Conversely, electrostatic interactions between carboxylate residues and divalent metal ions can impart reasonable structural stability to a protein. Calcium stabilizes staphylococcal nuclease[35] and increases the heat stability and reactivity of trypsin.[78] Many metallo-enzymes derive a good deal of their stabilization from specific metal coordination complexes.[22]

The role of inter- and intra-molecular ionic bonds among the α_{s1}-, β- and κ-caseins in stabilization of micelle structure is difficult to assess. Many potential sites for strong ion-pair bonds within an apolar environment exist, as deduced from consideration of the known sequences; and such bonds may play a role in micelle subunit interactions. Pepper *et al.*[61] demonstrated that carbamylation of 5 of 9 lysine residues of κ-casein destroyed the ability of κ-casein to stabilize α_{s1}-casein, thus demonstrating that ionic interactions may play a role in micelle structure. Furthermore, Hill[36] modified the arginine side-chains of the caseins and found differences in coagulation by rennin.

The estimated calcium content of milk is around 30 mM[19,105] far above the concentrations of Ca^{2+} required to precipitate the isolated α_{s1}- and β-caseins at room temperature.[90] The role of the phosphate residues in calcium binding has been investigated by the enzymatic dephosphorylation of α_{s1}-casein. Pepper and Thompson[60] and Bingham *et al.*[8] demonstrated that dephosphorylated α_{s1}-casein was still precipitated by calcium and showed decreased stabilization by κ-casein. The latter authors postulated that two nonphosphate calcium-binding sites occur in α_{s1}-casein, and it is the binding to these sites which induces precipitation of the dephosphorylated casein. Investigation of the κ-casein stabilized, dephosphorylated α_{s1}-casein by electron microscopy[8] showed larger but fewer micelle-like structures. In milks containing α_{s1}-A,[90,92] such large micelles are poorly solvated and less stable. Thus the formation of micelle-like structures is not totally dependent upon the formation of calcium-phosphate bonds between caseins; however, the resulting micelles may be less stable. Indeed, removal of calcium from micelles by chelating agents such as EDTA and fluoride[12] leads to disruption of casein micelle structure, as evidenced by electron microscopy.

The total number of charged groups of the casein monomers (Tables 9.1 and 9.2) reveals that in the formation of a casein micelle, not all

of these ionic groups can occupy a surface position. This would indicate either that much energy is used to bury these groups or that the structure is porous and available to the solvent, water. The latter proposition is born out by the experimental evidence. Ribadeau-Dumas and Garnier[65] noted that carboxypeptidase A is able to remove, quantitatively, the carboxyl-terminal residues from the α_{s1}-, β- and κ-caseins of native micelles, demonstrating that this enzyme (M.W. 40,000) is able to penetrate into the center of the casein micelle. Thompson *et al.*[90,92] have shown that the casein micelle is a highly solvated structure with an average of 1.90 gm water per gm protein. They also noted (Fig. 9.2) a strong positive correlation between the degree of solvation and heat stability.[92] The degree of solvation of the micelle, and hence the heat stability of the milk, hinges upon a variety of factors[62,68,92] not the least of which is the calcium:phosphate ratio. Increases in the calcium content of milk causes decreased heat stability,[62,68,99] possibly by altering the degree of solvation of the casein micelle. Thus the micelle emerges as a highly solvated porous structure. Environments which tend to decrease solvent interaction lower the stability of the micelle, which in turn destabilizes the milk. These interactions relate back to the proposition that the ionic residues of the individual casein



From Thompson et al.[92]

FIG. 9.2. PLOT OF THE SOLVATION OF THE CASEIN PELLETS (GRAMS WATER/GRAM PELLET) VERSUS HEAT COAGULATION TIME IN SECONDS

Dotted lines represent 95% confidence limits.

monomers cannot be totally buried, but must be exposed to solvent.

The better early measurements of the monomer molecular weights of the isolated casein fractions were obtained at pH 11 to 12.[50,102] At these pH values the positively charged lysine residues and a portion of the arginine residues have been neutralized, thus increasing the charge repulsions of the carboxyl and phosphate residues. However, prolonged exposure to high pH may produce degradation, as pointed out by Noelken.[55] These same effects operate in the casein micelle; as the pH of milk is brought to 11 to 12, the micelle structure is disrupted, with accompanying changes in turbidity and viscosity. Presumably, exposure to high pH for long periods of time, for example, in the production of sodium caseinate, may cause degradation, and hence alter the characteristics of the product.

Hydrogen Bonding Secondary and Tertiary Structure.—Many globular proteins, such as myoglobin, are stabilized by a high degree of α-helical structure.[9] In addition to the fibrous proteins, the so-called β- or pleated sheet structure has been detected by X-ray crystallography in globular proteins, notably lactate dehydrogenase[35] and others.[9,26] These secondary structures are stabilized by the formation of hydrogen bonds along the polypeptide backbone. Many proteins have been shown to contain significant amounts of secondary structure, as determined by spectral methods, such as circular dichroism, optical rotatory dispersion, and infrared spectroscopy.[95] However, in at least one case these methods have proved inaccurate in predicting the amount of secondary structure of a protein.[5] Therefore, the spectral methods can provide a good estimate of the amount of secondary structure, but they are subject to error. In many cases, then, some degree of stabilization is achieved by the formation of α-helical or β-structure, but not all stable proteins contain considerable amounts of these conformations. Other bonding forces (noted above) and perhaps even "sterically restricted" random structures may contribute significantly to the stabilization of a protein. The formation of α- and β-structures is also highly dependent upon the amino acid side-chains (proline, for example, breaks helical structures). In fact, the state of ionization of the side-chains and the solvent used play a role in the formation of α-helix.[9,26]

Spectral investigations of the isolated caseins have shown that these proteins possess little secondary structure. Herskovits[34] demonstrated by optical rotatory dispersion, using Moffit-Yang, Drude and Shechter-Blout analyses, that in aqueous solutions neither the individual casein components (α_{S1}-, β- or κ-) nor whole sodium caseinate exhibit an appreciable degree of α-helical content. Noelken and Reibstein[56] concluded that β-casein exhibits a random coil conformation in both aqueous solution and in 6*M* guanidine-HCl. Evidence has been

accumulated[58,73,82,104] that α_{s1}- and β-caseins are intermediate between a totally random and a globular protein in conformation. The above observed properties of the caseins are in good agreement with the high incidence of proline scattered throughout the α_{s1}- and β-caseins as derived from analyses of their sequences. Since little or no secondary structure occurs in the individual casein components, one would expect that the degree of stabilization contributed to the casein micelle by α-helix or β-structure would be quite low.

Theoretically, hydrogen bonds between ionizable side-chains accessible to the solvent, water, contribute to a limited degree to the stabilization of monomeric proteins.[9,44] These groups are already hydrogen-bonded to water and the water-residue hydrogen bond would have to be broken before a residue-residue hydrogen bond could be formed. Nevertheless, once two subunits of a protein begin to interact, these surface groups may no longer be totally hydrated, and hydrogen bonds could form between monomers as a result of the altered environment.

Hydrogen bonding between casein monomers in the casein micelle may occur. Subunit interactions, at present, have not been sufficiently detailed by crystallographic evidence to support or rule out these types of bonds, but some intra-chain hydrogen bonds do occur in monomeric proteins.[9] It is also possible that some hydrogen bonding may occur in the self-association[73,75] of α_{s1}-casein. Certainly, in the formation of the highly aggregated casein micelle, such bonds between the various casein components would be possible.

The Role of Disulfide Bonds.—The folding about of helical segments, pleated sheet areas and unordered structures of a polypeptide chain is referred to as tertiary structure. The tertiary structure of proteins can be locked in place by the formation of disulfide bonds between distal cysteine residues. In fact, non-identical polypeptide chains can be held together by disulfide bonding, as in the case of γ-globulins. Evidence has been presented that for several proteins,[9] the disulfide bridges do not cause the formation of secondary and tertiary structure, but tend to stabilize the preformed conformations. Proteins such as lysozyme and RNase with a relatively high degree of disulfide bonding are quite stable, but not all stable proteins necessarily contain disulfide bonds.

As noted above, κ-casein is the only major component of the casein-protein complex that contains cystine (or cysteine). The occurrence of free sulfhydryl groups in the native casein complex has been reported by some workers,[3] but not by others.[41] Hence, the degree of disulfide cross-linkages, which normally occur in the casein micelle, is difficult to estimate. Swaisgood and Brunner[80] reported that a good approximation of the minimum size of κ-casein would be a disulfide-linked trimer,

but for the most part their evidence[80,81] would indicate a greater degree of cross-linking. However, Woychik *et al.*[106] demonstrated that reduced and alkylated κ-casein stabilized α_{s1}-casein against calcium precipitation as well as native κ-casein. It appears that while the disulfide bridges of the casein micelle may contribute to the overall stability of the casein micelles, they are neither the driving force for micelle formation nor the central feature of the formed micelle.

Colloidal Calcium Phosphate.—The total calcium content of skimmilk has been estimated to be 30 mM,[19,105] but the calcium-ion content of serum, prepared by ultrafiltration or centrifugation of skimmilk is only $\sim$ 2.9 mM.[10,19] Specific ion electrode studies yield a value of 2.5 mM calcium (II) for skimmilk.[20] Thus, more than 90% of the calcium content of skimmilk is in some way associated with the casein micelles. Subsequent washing of the micelles removes only a small portion of the calcium and other salts. The mineral contents of washed micelles prepared by centrifugation[19] and of "primary micelles" prepared by gel filtration[10] are compared in Table 9.3; both methods appear to yield similar calcium and phosphate contents. The existence of this so-called "colloidal calcium phosphate" was postulated as early as 1915 by Van Slyke and Bosworth,[100] who concluded that the nonprotein-bound colloidal calcium phosphate was present in a 1:1 molar ratio which approximates discalcium phosphate. Later workers[19,105] have calculated that the colloidal calcium phosphate more closely resembles tri-calcium phosphate with a $Ca:PO_4$ molar ratio of 1.5. Calculation

Table 9.3

TOTAL MINERAL COMPOSITION OF CASEIN MICELLES
mMOLES/100 GM CASEIN[a]

	Washed Micelles[b] by Centrifugation	Micelles by Gel Filtration[10]	Unwashed Micelles by Centrifugation[b]	Unwashed Micelles by Gel Filtration[10]
Calcium	69.6 64.1	68.9	71.0	79.0
Magnesium	4.2	3.3	4.5	6.9
Sodium	4.5			
Potassium	6.2	6.2		
Casein (PO_4)	22.2 23.2	28.2		
Inorganic (PO_4)	28.9 27.8	21.8	47.8	43.2
Citrate	1.6	0.0	6.2	4.7

[a] Casein N × 6.4.
[b] Adapted from Table 70, McMeekin and Groves, Chapter 9, "Fundamentals of Dairy Chemistry," 1st Edition.

of such a ratio after subtracting casein-bound calcium is subject to inherent error. Binding studies[21] on the isolated β- and κ-caseins show a good 1:1 correlation between calcium ions bound and phosphate residues, while α_{s1}-casein appears to have[8,21] 1 to 2 nonphosphate calcium binding sites. The application of these results allows the calculation of a Ca/PO_4 molar ratio from Table 9.3. The ratio obtained for washed micelles and micelles prepared by gel filtration are 1.6 and 1.8, respectively. The latter value differs from that calculated by Boulet *et al.*[10] because they assumed a 2:1 casein phosphate:calcium ion ratio. Thus, attempting an exact assignment of calcium to either the casein fraction or the colloidal calcium phosphate fraction can cause discrepancies in the calculated ratio. It must be realized that these data are average values based on average distributions of the caseins and the minerals. Not only do the mineral content and the casein distribution vary from one individual milk to another, but the various micelle fractions within a single sample are probably not of uniform composition.

It is clear from Table 9.3 that there are two distinct forms of ions associated with the casein micelle—an outer system, perhaps in the form of a charged double layer,[10] and an inner system not easily washed away. As noted above, the casein micelle is a highly porous, well solvated system, and the occlusion of ions within this network is not unexpected; however, some actual complex formation between the colloidal calcium phosphate and the casein cannot be ruled out. If one examines the pK's of phosphoric acid, it would seem most likely that the associating species of phosphate would be $(HPO_4)^{2-}$. Termine and Posner[85] studied the *in vitro* formation of calcium phosphate at pH 7.4, and concluded that an amorphous calcium phosphate phase (with a $Ca:PO_4$ molar ratio of 1.5) formed prior to the transition to crystalline apatite. In a subsequent study,[86] it was shown that casein and some other macromolecules enhanced the stability of the amorphous calcium phosphate and, in fact, retarded the amorphous $\rightarrow$ crystalline transition. It would appear then that conditions should favor the formation of an amorphous-calcium phosphate-caseinate complex in milk. The exact nature of this complex (or occlusion) is as yet undetermined, though its role in casein micelle stabilization is well documented.

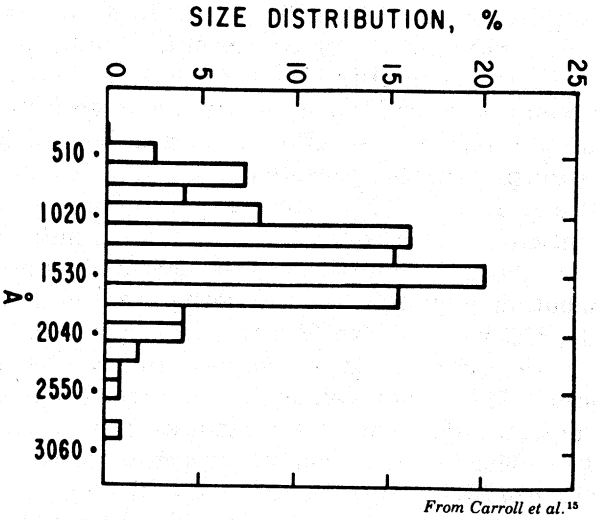
Pyne and McGann[63] demonstrated that the colloidal calcium phosphate content of milk decreases as the pH is lowered from 6.7 to 5.0 at 5°C. If a small sample of pH 5.0 milk is then dialyzed at 5°C against several large volumes of the original milk, the pH returns to 6.7, but the colloidal calcium phosphate is no longer present. Milk brought to essentially zero colloidal calcium phosphate concentration at pH 5 and dialyzed back to 6.7 in this manner has been termed

colloidal calcium phosphate-free milk (CPF milk). In a later study McGann and Pyne[48] investigated the properties of CPF milk as compared to the original nontreated milk. The CPF milk is translucent as compared to ordinary milk, and has a greatly increased viscosity. Addition of Ca^{2+} up to $\sim 1M$ has little effect on normal milk at 25°C, provided the increase in pH is not compensated for. CPF milks, however, are precipitated at added calcium-ion concentrations of only 25 mM. There is no apparent difference between CPF and normal milks with regard to the primary phase of rennin attack as measured by release of soluble nitrogen but, interestingly, the CPF milks are slightly more heat-stable. Finally, McGann and Pyne[48] noted that, at low temperatures, β-casein is more firmly bound to rennin-clotted normal milk than to rennin-clotted CPF milks. Jenness *et al.*[39] noted a marked increase in serum or nonmicellar casein, accompanied by an increased translucence as the colloidal calcium phosphate content of milk was reduced by the addition of EDTA. Rose[69] noted that, while Ca^{2+} addition generally decreases the serum casein content of milk, lowering the pH of milk to 5.3 and the subsequent release of Ca^{2+} actually increase the serum casein content. This result led Rose[69] to speculate that the colloidal calcium phosphate aids in maintaining micelle stability. CPF milks and normal milks were compared by Downey and Murphy[23] with respect to their elution volumes on gel chromatography (Sepharose 2B) in a synthetic milk serum. The normal casein micelles eluted at V_o yielded a molecular weight of $> 10^8$, but CPF micelles eluted at a volume consistent with a molecular weight of $\sim 2 \times 10^6$. However, this result could also be explained by a marked change in shape (frictional ratio).

All the discussion presented above indicates that colloidal calcium phosphate is involved in maintaining the structural integrity of the casein micelle. Occlusion of amorphous apatite or possible complexation of the mineral must occur, but the exact mechanism by which stabilization is achieved is as yet unknown.

Casein Micelle Structure

From the above discussion one might conclude that the individual caseins have been studied in sufficient detail to yield an intimate knowledge of the structure of the casein micelle. This is not the case, and nearly as many models have been proposed as there are investigators. Let us briefly consider why this situation exists. Electron microscopy (Fig. 9.3) of the casein micelles of bovine milk indicates an average diameter of ~1,400 Å for the spherically shaped micelles. Thus, the volume occupied by a micelle would be of the order of $\sim 1.4 \times 10^9$ A^3. For comparison, the β-lactoglobulin monomer[96] occupies a volume



From Carroll et al.[15]

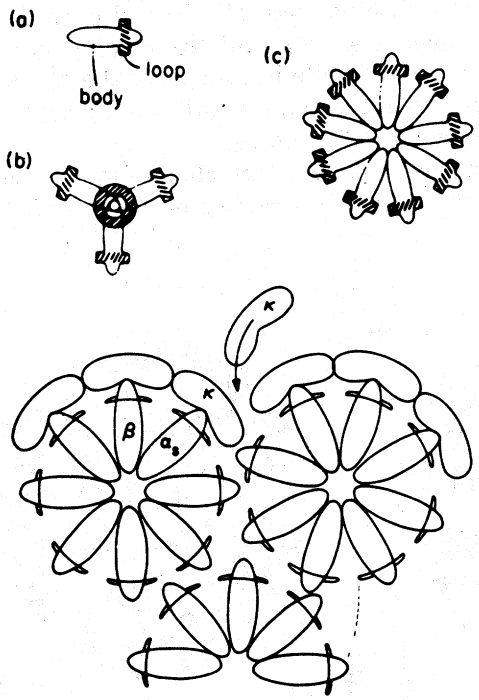
FIG. 9.3. DETERMINATION OF THE SIZE DISTRIBUTION OF GLUTARALDEHYDE-FIXED CASEIN MICELLES FROM SKIMMILK

of ~2.4 × 10^4 Å^3. Theoretically, more than 50,000 β-lactoglobulin-like monomers could be arranged into a sphere the size of a casein micelle. Molecular weight measurements for the micelle range from 10^7 to 10^9.[15,23] A speculative calculation, based on 23,000 average M.W. for the casein monomers $[(3\alpha_{s1} + 2\beta + 1\kappa)/6]$ and employing only 25,000 monomers yields a micelle molecular weight of 6×10^8. This would indicate low-density packing of the casein monomers, which is consistent with the high hydrations, the random structures and the high negative charge densities of the caseins, as compared to β-lactoglobulin. It is therefore understandable that the mechanism of assembly of this aggregate of around 25,000 monomers has not been fully elucidated. For the purpose of discussion, we shall group the various proposed models into three classes.

Coat-Core Models.—The first class of models to be discussed actually contains two diametrically opposed theories. The model proposed by Waugh and his co-workers[70,104] is primarily based upon their studies of the solubilities of the caseins in Ca^{2+} solutions. The model, in essence, describes the formation of low weight ratio complexes of α_{s1}- and κ-casein in the absence of calcium. Upon addition of calcium ion, the α_{s1} - or β-caseins, depicted as monomers with a charged phosphate loop in Fig. 9.4a, begin to aggregate to a limiting size (the caseinate core). In the presence of the low weight ratio α_{s1}-κ-complexes, precipitation of the casein is prevented by the formation of a monolayer of these low-weight α_{s1}-κ-complexes which envelops the core aggregates. This coat has the κ-casein monomers spread out on the surface, and the

micelle size is therefore dictated by the amount of κ-casein available. In the absence of κ-casein, the α_{s1}- and β-cores agglutinate and precipitate from solution. Waugh's model, as presented in Fig. 9.4a, has a good deal of appeal since it explains the lyophilic nature of the colloidal casein complex, as well as the ready accessibility of κ-casein to the enzyme rennin.

Parry and Carroll[57] attempted to locate this surface κ-casein proposed by Waugh by use of electron microscopy. Using ferritin-labeled anti-κ-casein-immunoglobulins, they investigated the possiblity of surface κ-casein and found little or no concentration of κ-casein on the surface of the casein micelles. Based on these results, and the size of the isolated κ-casein complex, Parry concluded that the κ-casein might serve as a point of nucleation, about which the calcium-insoluble caseins might cluster and subsequently be stabilized by colloidal calcium phosphate



Adapted from Rose[70]

FIG. 9.4a. WAUGH'S PROPOSED MODEL FOR THE CASEIN MICELLE

(a)—Monomer model of α_{s_1}- or β-casein with charged loop. (b)—A tetramer of α_{s_1}-casein monomers. (c)—planar model of a core polymer of α_{s_1}- and β-caseins. The lower portion shows how κ-casein might coat core polymers.

(see Fig. 9.4b). The action of rennin on the micelles was accounted for by demonstrating that serum κ-casein can participate in coagulation and may be involved in the formation of bridges between micelles.

The models of Parry and Waugh both predict a nonuniform distribution of κ-casein and in a sense are based upon nucleation about a core (Parry's core = κ-casein; Waught's core = α_{s1}-, β-calcium caseinate). It is important to note that both models predict no particular stoichiometry for the casein components and demonstrate no subunit structures composed of all three casein components. Secondly, Waugh's model does not incorporate any colloidal calcium phosphate which, as noted above, plays an important role in casein micelle structure and stability.

Finally, Ashoor *et al.*[2] have recently demonstrated that papain which had been cross-linked by glutaraldehyde into a large insoluble polymer caused proteolysis of all three major components of isolated casein micelles. The α_{s1}-, β- and κ-caseins were all cleaved proportionately by the enzyme superpolymer. Therefore, all three components must occupy surface positions on the micelle in relatively the same proportions in which they occur in milk. This result would seem to rule out any preferential localization of κ-casein.

Internal Structure Models.—The second class of models to be discussed are based upon the known properties of the isolated casein

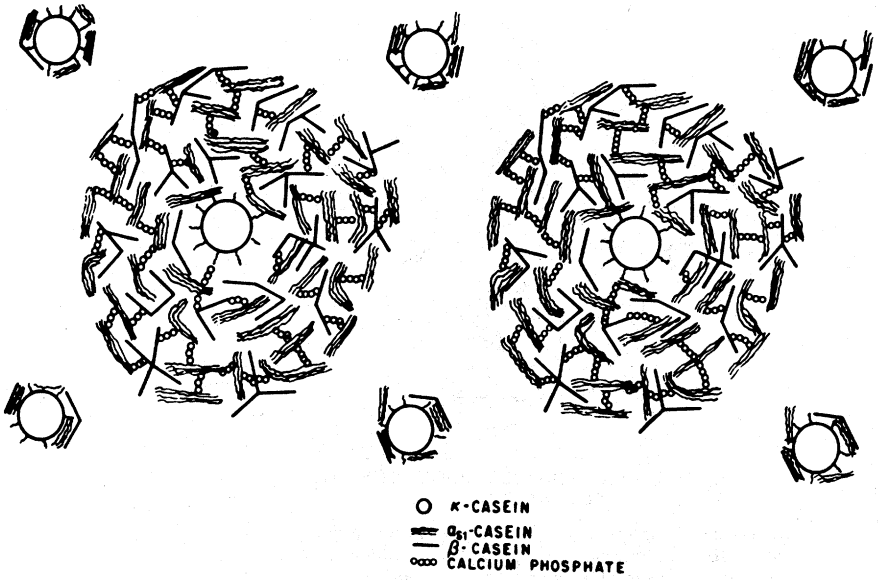
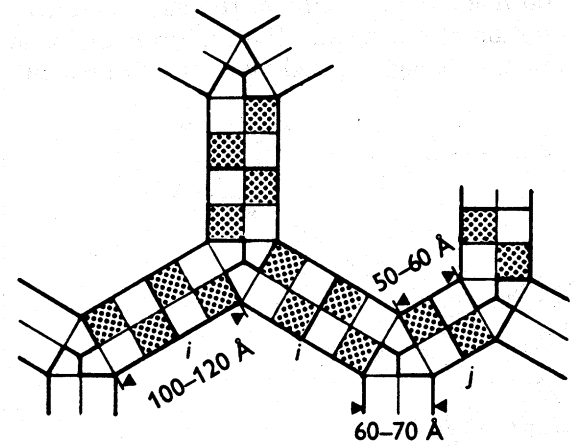


FIG. 9.4b. CASEIN MICELLE MODEL PROPOSED BY PARRY AND CAROLL[57] DEPICTING THE LOCATION OF κ-CASEIN IN THE MICELLE

components, which in turn cause or direct the formation of the internal structure of the casein micelle.

Garnier and Ribadeau-Dumas have proposed a model for the casein micelle[27] which places a good deal of emphasis on κ-casein as the keystone of micelle structure. Trimers of κ-casein are linked to three chains of α_{s1}- and β-casein which radiate from the κ-casein node (a Y-like structure), as shown in Fig. 9.5a. These chains of α_{s1}- and β-casein may connect with other κ-nodes to form a loosely packed network. Garnier and Ribadeau-Dumas favor this type of network because it yields an open, porous structure, and they have demonstrated[65] that carboxypeptidase-A with a molecular weight of $\sim$40,000 is able to remove the C-terminal amino acids of all the casein components. The model satisfies the demonstrated porosity, but places great steric restraints upon κ-casein, which possesses no α-helical or other prominant secondary structures. In addition, studies by Cheeseman[17] and others[80,81] indicate that while disulfide-linked trimers of κ-casein do occur, the majority of the κ-casein may form aggregates of higher as well as lower orders. Finally, the model assigns no definite role to calcium caseinate interactions, and ignores the possibility of colloidal calcium phosphate involvement in stabilization of the micelle.



(f)

α_{s1} casein

β casein

κ casein

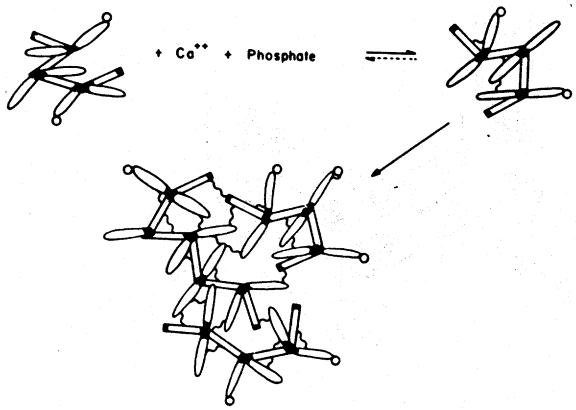
Adapted from Garnier and Ribadeau-Dumas[27]

FIG. 9.5a. STRUCTURE OF THE REPEATING UNIT OF THE CASEIN MICELLE

Rose used the known endothermic polymerization of β-casein as the basis for his micelle model.[70] In this model β-casein monomers begin to self-associate into chain-like polymers to which α_{s1}-monomers become attached (Fig. 9.5b), and κ-casein in turn interacts with the α_{s1}-monomers. The β-casein of the thread is directed inward, the κ-outward, but as these segments coalesce, a small amount of κ-casein is inevitably placed in an internal position. As the micelle is formed, colloidal calcium phosphate is incorporated into the network as a stabilizing agent. The model is appealing in that it accounts for the occurrence of some overall stoichiometry of the various casein components, while demonstrating the role of colloidal calcium phosphate in micelle stabilization. The choice of β-casein as the basis for micelle formation is, however, questionable since Waugh *et al*[104] have shown that α_{s1}- and β-caseins tend to form mixed polymers randomly; secondly, β-casein is quite structureless in solution; and finally, synthetic micelles can be formed from simple α_{s1}- and κ-casein complexes in the absence of β-casein.

Subunit Models.—The final class of models to be discussed is that which proposes subunit structure for the casein micelle. Shimmin and Hill[77] first postulated such a model based upon their study of ultrathin cross-sections of embedded casein micelles by electron microscopy. They predicted a diameter of 100 Å for the subunits of the casein micelle.

Morr[53] studied the disruption of casein micelles and proposed that the α_{s1}-β- and κ-monomers may be aggregated by calcium into small



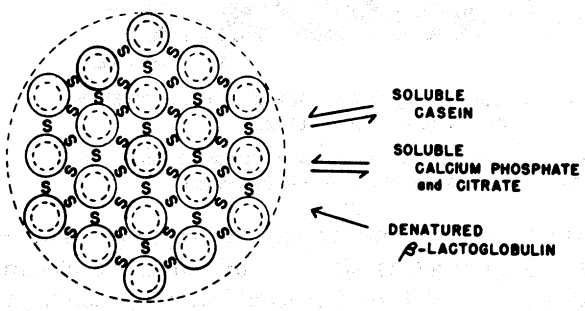
Adapted from Rose[70]

FIG. 9.5b. SCHEMATIC REPRESENTATION OF THE FORMATION OF A SMALL CASEIN MICELLE

The rods represent β-casein, the more eliptical rods represent α_{s1}-casein and the S-shaped lines depict apatite chain formation. The circles represent κ-casein.

subunits in much the same fashion as Waugh[104] had suggested for the entire micelle. Morr's subunits, as estimated by sedimentation velocity, have a diameter of ~ 300 Å. The subunits are stabilized by hydrophobic bonding and calcium caseinate bridges, and these subunits, in turn, are aggregated into micellar structure by colloidal calcium phosphate. Morr's model is summarized in Fig. 9.6. The average subunit size, postulated by Morr, is somewhat larger than that of Shimmin and Hill.

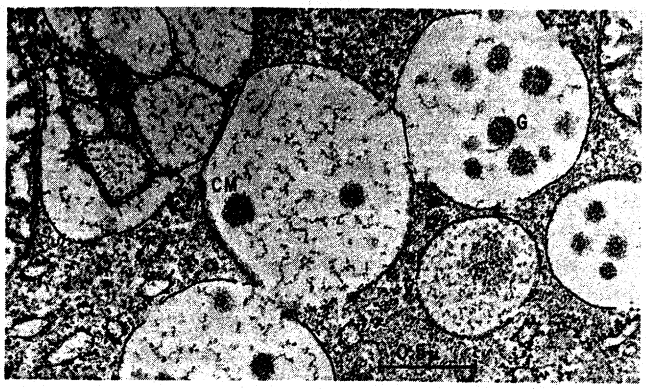
The hypothesis of Shimmin and Hill,[77] that sections of the casein micelles contain particles of ~100 Å in diameter, was invoked by Carroll *et al.*[13] and by Farrell and Thompson,[24] who observed by electron microscopy particles of ~100 Å diameter in the Golgi vacuoles of lactating rat mammary gland. These particles were uniform in size and appeared to form thread-like structures which, in turn, coalesced into the spherically shaped casein micelle (Fig. 9.7a, b). Subsequently, Beery *et al.*[4] reported similar observations in bovine mammary tissue. The biosynthesis of the casein micelle from small subunits was correlated with the disruption of casein micelles by dissociating agents by Carroll *et al.*[12] Using EDTA, urea, sodium lauryl sulfate, and sodium fluoride to disrupt micelles, the latter workers found particles of ~ 100 ± 20 Å diameter; and they noted that micelle assembly from subunits should lead to a rather uniform distribution of α_{s1}-, β- and κ-caseins both on the surface and in the interior of the casein micelle. Schmidt and Bucheim[74] dialyzed milk free of calcium in the cold and also used high pressure to disrupt casein micelles; in both cases they obtained subunits of 100 Å diameter. Subsequently, Pepper[59] reported a Stokes radius of ~50 Å for first-cycle (Ca^{2+} -free) casein as determined by gel filtration. The first-cycle casein, after gel filtration,



After Morr[70]

FIG. 9.6. STRUCTURE OF THE CASEIN MICELLE

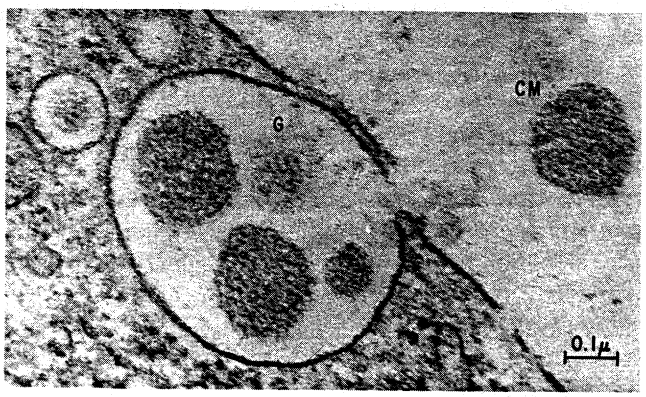
The S-shaped lines represent calcium phosphate linkages between small spherical complexes of the α_{s1}-, β-, and κ-caseins.



From Carroll et al.[13]

FIG. 9.7a. FORMATION OF CASEIN MICELLES (CM) WITHIN GOLGI VACUOLES (G) OF LACTATING RAT MAMMARY GLAND

Initially, thread-like structures with some degree of periodicity appear, then more compact micelles seem to occur. Sections of the gland were fixed in buffered OsO_4, Epon embedded, and stained with uranyl acetate and lead citrate.



From Carroll et al.[13]

FIG. 9.7b. A GOLGI VACUOLE ABOUT TO DISCHARGE ITS CONTENTS INTO THE ALVEOLAR LUMEN

The golgi vacuole shown appears to impinge upon the plasma membrane. A casein micelle is already present in the lumen.

contained qualitatively all the major casein fractions. Therefore, the question yet to be resolved is whether or not these casein micelle subunits observed by all the above exhibit any stoichiometry in terms of their α_{s1}-, β-, and κ-casein content.

It has long been recognized that at least the α_{s1}-and κ-casein components occur in close association in the "α-casein complex," β-casein being more loosely connected to the micellar complex. Furthermore,

the total micellar casein exhibits an overall ratio of 3 α_{s1}-:2β-:1 κ-casein. The apparent uniformity of first-cycle (Ca^{2+}-free) casein and the subunits of the Golgi vacuoles would favor some consistent stoichiometry, but there exists the reported correlation between micelle size and κ-casein content[69,71,104] which would argue against uniform subunit composition. Thus, the existence of some type of subunit structure appears certain, and the question to be decided now is the nature of these reported subunits.

From the biosynthetic point of view, the build-up of the micelle from subunits is quite attractive, as it brings the casein components into the region of assembly with minimal interactions. Addition of calcium ion could cause the polymerization of casein subunits into longer chains, which could be stabilized into micellar spheres by the deposition of colloidal calcium phosphate. The assembly of the casein micelle from preformed subunits need not be as specific as the mechanism of assembly of tobacco mosaic virus. In the latter case, its structured RNA core[11] plays a vital role in directing correctly the assembly of the virus particle from its preformed subunits, whereas in the case of the micelle, amorphous apatite apparently serves this function. In attempting to solve the problem of casein micelle structure, it should be born in mind that the biological function of the micelle is efficient nutrition. Hence, the interactions which yield this product, the casein micelle, need not be as specific as those which result in the formation of a virus or an enzyme.

Proteolytic Action and Micelle Models.—The action of rennin on the casein micelle is primarily hydrolysis of the highly sensitive phenylalanine-methionine peptide bond of κ-casein. Sequence data[40] show that 3 to 8 residues from this bond in either direction is the unusual sequence proline-proline, which perhaps accounts for the high susceptibility of this specific bond. Splitting of the bond[42] causes formation of the rather insoluble para-κ-casein and the highly hydrophilic (and carbohydrate-rich) peptide termed GMP or MP (glycomacropeptide or macropeptide). As a result of the action of rennin, the micelles coagulate or clot. All the casein micelle models must account for this phenomenon.

Waugh's model solves this problem most readily; since all the κ-casein is on the surface, the cleavage of GMP results in the loss of charge repulsions which normally prevent coagulation. Parry pointed out the possible role of serum κ-casein in the rennin reaction, and Garnier and Ribadeau-Dumas noted the possibility that rennin could penetrate a highly porous system. Recently, Ashoor *et al.*[2] have shown that papain, which had been insolubilized by glutaraldehyde cross-linkages, caused proteolysis of all three major components of casein micelles, demon-

strating that not only κ- but α_{s1}- and β-casein occupy surface positions. Because of the high incidence of apolar residues in all the caseins and the large number of monomers, it is inconceivable that all the apolar chains would be buried and, therefore, many hydrophobic patches probably exist on the surface of the micelle. Normally, coagulation is prevented by the charged surface groups contributed by all three components. When a sufficient number of the κ-casein-GMP fragments have been removed by rennin action, coagulation or clotting could occur, with the addition of serum para-κ-bridges, as postulated by Parry.[57] In addition, internal hydrolysis of all casein components (and this too occurs) would cause the porous micelle to dehydrate on loss of internal macropeptide, leading to additional destabilization of the system. This latter phenomenon may be as important as those occurring at the surface.

What has been noted above in regard to the specific enzyme rennin would also be true of any protease which would be added to, or naturally occur in, milk. Chen and Ledford[18] have studied the "milk protease" and found it to be trypsin-like. Limited proteolysis by this or by other as yet uncharacterized proteases or their reactivation upon storage may be the cause of many of the problems encountered in the storage of processed whole or concentrated milks and dairy products.

THE MILK SERUM PHASE

The Serum Proteins

The proteins of the dispersed phase of milk, the casein micelles, account for up to 74% of the total protein of skimmilk (Table 9.4). The serum or whey proteins are those which remain in solution after

Table 9.4

AVERAGE COMPOSITION OF WARM SKIMMILK PROTEIN[a]

	gm/100 gm Milk[b]	% Total Protein[b]
Colloidal casein	2.36	74
Serum casein	0.26	8
β-Lactoglobulin	0.29	9
α-Lactalbumin	0.13	4
Bovine serum albumin	0.03	1
Total immunoglobulins	0.06	2
Other proteins	0.06	2

[a] Averaged from data of several sources.[69,71,72,93]
[b] All values normalized to 3.2 gm total protein/100 gm milk.

the micelles are removed; these proteins are not incorporated into colloidal complexes. Depending upon the method of removal of the casein micelles, varying amounts of serum casein will be included in this fraction. The major noncasein serum proteins are: β-lactoglobulin, α-lactalbumin, bovine serum albumin and the milk immunoglobulins.[72]

β-Lactoglobulin.—β-Lactoglobulin is the major serum protein; it accounts for up to 50% of the noncasein protein of skimmilk and exhibits quite unique structural features. β-Lactoglobulin has a monomer molecular weight of 18,000 daltons; Frank and Braunitzer[25] have published a rather complete amino-acid sequence for the molecule. Although X-ray crystallography[30] has been done on β-lactoglobulin, high resolution data has not been obtained. However, from physical data and chemical modification experiments, much has been deduced concerning the topography of the molecule,[99] as well as its rather unique mode of association. The 18,000 dalton monomer occurs only below pH 3.5 and above pH 7.5. Between pH 3.5 and pH 7.5, β-lactoglobulin exists as a dimer with a molecular weight of 36,000; its conformation has been well characterized.[98] At reduced temperatures, between pH 3.7 and 5.1, the dimers of the β-lactoglobulin A genetic variant form a specific octamer with 422 symmetry.[96] β-Lactoglobulin B does not undergo this self-association to an appreciable extent, and the C genetic variant does not octamerize at all.[96,98] The conformational transitions, which bring about these associations, have been well documented, and the analogy of these interactions to allosteric control mechanisms has been noted.[97] The precise biological function of this molecule is unknown, though several hypotheses have been advanced.[24]

Because of its ready availability and unique properties, β-lactoglobulin has been used as a model protein in many studies. However, the major noncasein protein of milk, β-lactoglobulin, also plays an important part during processing as the result of two of its features. In the first place, β-lactoglobulin has well-defined secondary, tertiary and quaternary structures which are susceptible to denaturation. Secondly, one free sulfhydryl group occurs per 18,000 monomer.[46] Thus the integrity of the β-lactoglobulin molecule must be retained in part, in order to prevent coagulation as the result of irreversible denaturation; and conditions which affect free sulfhydryl groups or cause disulfide interchange with other proteins must be avoided.

Protein denaturation has been reviewed by Tanford.[84] Environmental influences that cause reversible denaturation, when carried to an extreme, generally produce some irreversible denaturation (see Coagulation Chapter 11). In discussing the phenomenon of denaturation, Tanford[84] outlined the contributions of conformational, inter-chain,

protein-solvent, and electrostatic interactions to the free energy of the transition from the native (N) to the denatured (D) state. He considered both the influence of environmental factors, such as temperature and pressure, and also of binding effects on the N → D transition. Increases of temperature and pressure, as well as the binding of denaturants (e.g., guanidine, urea, and detergents), the binding of inorganic ion, and the binding of hydrogen ion (pH) may facilitate the transition to the denatured state. Tanford further notes that each type of denaturing agent leads to a particular denatured state. In each case, the resulting polypeptide chain has more exposed apolar groups, which increases the probability of aggregation unless these areas are stabilized by the solvent (e.g., urea and guanidine). Denaturation in aqueous solution often leads to aggregation and, finally, precipitation or coagulation. It is this latter event which one wishes to prevent in the processing of dairy products. Carroll *et al.*[14] demonstrated by the use of electron microscopy that denaturation and subsequent precipitation of whey proteins is the basis for the gelation of high-temperature, short-time, sterilized, concentrated milk. Hence, it would appear that environmental influences, which tend to preserve the native structure of β-lactoglobulin and the other whey proteins, would favor stability; conversely, denaturation and subsequent coagulation would lead to instability. Factors which influence the denaturation of β-lactoglobulin have been reviewed by Tilley[94] and McKenzie.[49] Generally speaking, heat, pH above 8.6,[94] and increased calcium ion concentration[108] tend to increase the N → D transition of β-lactoglobulin.

A second source of instability in β-lactoglobulin is its unique sequence[24] -Cys-Cys-. It has been reported[49] that residue 69 is the free sulfhydryl group of β-lactoglobulin, while residue 70 is involved in a disulfide bond. Hence, exposure to strong base or heat may promote disulfide interchange and lead to denaturation. McKenzie[49] has summarized the work of several groups who have demonstrated that sulfhydryl blocking reagents increase the stability of β-lactoglobulin. The free sulfhydryl group of β-lactoglobulin has been implicated in the formation of complexes with κ-casein upon heating.[109] Townend *et al.*[99] pointed out that in the native state this residue is partially buried; hence, some degree of denaturation must occur in order to totally expose this sulfhydryl residue. Since molecules such as carboxypeptidase and myoglobin[65] can penetrate the casein micelle, β-lactoglobulin may likewise do so, thus inducing formation of disulfide-bonded complexes with κ-casein upon heating. Such complexes would alter the stability of the casein micelle.

α-Lactalbumin.—The best-characterized milk protein is α-

lactalbumin. Gordon[28] has recently reviewed the properties of α-lactalbumin, which accounts for up to 25% of whey protein and $\sim$4% of total milk protein (Table 9.4). α-Lactalbumin has been assigned a unique biochemical role as the specifier protein of the lactose synthetase system. The complete sequence of α-lactalbumin is known[101] and the molecule exhibits a strong structural relationship to lysozyme. α-Lactalbumin is quite stable, with 4 disulfide cross-links for a monomer molecular weight of 14,000. Kronman[45] has demonstrated that α-lactalbumin is denatured at low pH and then undergoes an association reaction, which requires somewhat elevated protein concentration and may not occur in milk products. However, in whey concentrates, acid denaturation of α-lactalbumin may play an important role in the loss of its functional properties. Hunziker and Tarassuk[38] have reported that the free sulfhydryl of β-lactoglobulin can promote complex formation with α-lactalbumin through disulfide reactions.

Other Serum Proteins.—Serum albumin and immunoglobulins[72] occur in skimmilk to a limited extent; in conjunction with various enzymes,[76] they may account for up to 4% of total milk protein. All these proteins contain a significant amount of native structure and are susceptible to various forms of denaturation. In fact, limited protein denaturation is a desired result in terms of the required inactivation of many of the enzymes which have been noted to occur in milk. The reactivation of proteolytic and other enzymes, upon storage of processed dairy products, is undoubtedly the source of many problems. Hence, it appears as though conditions must be controlled so as to adequately inactivate enzymes, and reduce bacterial contamination without causing severe denaturation and consequent coagulation of the major whey proteins.

The Serum Caseins.—Not all the casein secreted by the lactating mammary cells is incorporated into casein micelles. Rose[69] and Downey and Murphy[23] have studied the occurrence and distribution of the serum caseins. Rose[69] found that in warm milk the serum caseins, on the average, account for about 10% of the total casein, and the serum casein contained all the major components of the micelle in varying proportions, but always in the order β->κ->α_{s1}-casein. In cold milks the percentages of β- and κ-casein in the serum increase as the serum casein increases. Rose has concluded that serum casein does not appear to be in true equilibrium with micellar casein,[69] and this conclusion has been supported by Downey and Murphy[23] and Boulet *et al.*[10]

Serum caseins may play an important role in the destabilization of casein micelles. The serum caseins are not stabilized by colloidal calcium phosphate; they would be readily attacked by proteolytic

enzymes and would be more susceptible to other environmental factors. The serum caseins would also be more likely to interact with β-lactoglobulin or other whey proteins. Parry and Carroll[57] have proposed that serum κ-casein is more readily attacked by rennin and plays an important role in the rennin clotting reaction.

Salt Content of Milk Serum

The salt content of milk serum, separated by several methods, has been determined by Davies and White.[19] Their average results for two milks, obtained by separating milk serum by diffusion, centrifugation, and clotting with rennet, are compared in Table 9.5. These values are in essential agreement with each other and with the very extensive previous results on the composition of the nonprotein aqueous phase of milk by the same authors.[105] Recently,[20] calcium ion-specific electrodes have been used to determine the free Ca^{2+} concentration of skimmilk.

Table 9.5

DISTRIBUTION OF SALTS (IN MG/100 GM MILK) BETWEEN DISSOLVED AND COLLOIDAL STATE IN MILK[a]

	Dissolved				Colloidal State
	Total in Milk	Diffusate (20°C)	Rennet Whey	Centrifuged Serum	(Average 3 Methods)
Total calcium	114.2	38.1	39.9	40.9	74.6
Ionized calcium	10.3[c]	11.7	11.6	11.9	—
Magnesium	11.0	7.4	7.8	8.1	3.3
Sodium	50	46	47	47	3.3
Potassium	148	137	143	141	8.0
Total phosphorus	84.8	37.7	37.4	37.9	47.1
Inorganic phosphorus	—	31.8	30.8	31.8	—
Citric acid	166	156	152	154	12.0
Chloride	106	106.5	106.2	105.6	—
Total nitrogen	—	20.7	124.6	110.7	—
Casein nitrogen	364	0	21.6	6.8	—
Lactose[b]	4800	4800	4800	4800	—

[a] Adapted from Davies and White.[19]
[b] Average of two separated milks, corrected for bound water.
[c] By specific ion electrode calculated from the data of Demott.[20]

Equilibria Between the Colloidal and the Serum Phases

Because of the complex nature of the milk system, the question of whether or not true equilibria occur is difficult to assess. The Handbook of Chemistry and Physics defines equilibrium as " . . . . . the state of affairs in which a reaction and its reverse reaction are taking place at equal velocities, so that the concentration of reactants is constant." Such conditions, in terms of physical equilibria, must exist for some milk components, but do not occur for others. For the sake of discussion, let us assume that the casein micelle is a porous, highly hydrated complex, with some degree of subunit structure, and that the micelle contains colloidal calcium phosphate, and is surrounded by a double layer of ions. Clearly, the outer layers of ions can be in equilibrium with those of the solvent, and these ions are removed readily by gel filtration.[10] The bulk of the inorganic ions of milk are definitely not in equilibrium with the environment as they are occluded in the dispersed phase and are not readily dialyzed away[19,105] or removed by gel filtration.[10] The work of Rose[69] indicates that, at room temperature and above, the serum and micellar caseins are also not in equilibrium. However, at lower temperatures,[16,69,79] β-casein readily dissociates from the micelle and enters the serum phase, together with κ-casein and some α_{s1}-casein. These fractions could be in equilibrium because, as the milk is warmed, these caseins return to the colloidal phase; however, whether true microscopic reversibility occurs or not is questionable, as the opportunity for hysteretic effects is enormous in the milk system. Even at low temperatures, a large part of the β-casein, and most of the α_{s1}-and κ-caseins, are not removed from the casein micelle. The serum caseins, while they are apparently not in equilibrium with the micellar casein, are probably in equilibrium with the ions of the serum phase. The addition of calcium decreases the net amount of serum casein,[69] and added calcium has also been reported to decrease the heat stability of milk.[62] Hence, effects on the serum proteins may destabilize the entire system. Also, the serum proteins, such as β-lactoglobulin, must be in equilibrium with the ionic environment; again added salts appear to affect the stability of these serum proteins.[108] If the micelle is sufficiently porous to admit carboxypeptidase,[65] β-lactoglobulin and the other serum proteins should equilibriate within the micellar phase as well. The water of the milk system, as well as the lactose, should be in equilibrium between the dispersed and the serum phase, but some internal water of hydration may not be, as the occluded calcium phosphate undoubtedly affects the hydration, and hence the heat stability,[92] of the milk system.

Generally speaking, many equilibrium situations occur in skimmilk as well as several distinct systems that are not in equilibrium. The

innate stability of this biological fluid may depend on the correct balance between these states. Conditions which perturb the equilibrium states temporarily affect the milk system; but overall the nonequilibrium states, which include colloidal calcium phosphate, the serum caseins, and the water of hydration, appear to contribute most to the stability of milk. Hence, environmental influences which disrupt the nonequilibrium states tend to cause greater destabilization of milk.

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